Differentiation of the Species of the Genus Brucella*

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THE differentiation of the known species or varieties of Brucella and the mechanism of their behavior which serves to separate one from the other continue to be one of the important problems occupying the minds of those who are engaged in studying this group of microparasites and the reactions which they elicit from the host. It is now known that there are three species in the genus: *Brucella melitensis* (Bruce), *Brucella abortus* (Bang), and *Brucella suis* (Traum). The accumulating data indicate that they invade and produce disturbances in the tissues of as many species of animals as any other known pathogenic microbe. The desirability of being able to identify them with certainty is therefore obvious.

It is imperative that a satisfactory method or methods be available to determine if a strain coming from one species of host is entirely different from a strain coming from another. It is also important to know whether each of the species of Brucella is confined to one particular host or may have several hosts. This is especially important from the standpoint of human infections if their source is to be established more definitely. At present it appears to be established that one species of Brucella is common to the goat, one to the cow, and one to the hog, but this does not imply that each is confined always to a single host. It may occasionally be found in a variety of hosts. In the past, a strain recovered from the goat was called *melitensis,* if from the cow it was called *abortus,* if from the hog it was also called *abortus.* Yet, those from the hog were isolated under aerobic conditions and considered more pathogenic for guinea pigs than the strain common to cattle.

The fact that a given strain comes from a cow, a hog or a goat is not conclusive proof that it is *abortus, suis* or *melitensis.* Not until hundreds of strains from different animals in different countries have

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been studied with respect to their individual peculiarities, if such exist, will the relationship of Brucella to different hosts be known.

During recent years there have been no less than 10 methods proposed for the differentiation of the species of Brucella. Most of these have dealt with only 2 of the species, *Br. abortus* (Bang), and *Br. melitensis* (Bruce). Most of these have been thoroughly discussed before. Recently, 2 more methods of distinguishing *abortus* from *melitensis* have been proposed. One, by Favilli,[1] is based on the difference in the viability of the two on a semi-solid culture medium, and the other, by Valenti,[2] on the bactericidal action of homologous serums coming from individuals who have recovered from undulant fever.

The first would appear to possess too great a degree of uncertainty; yet, cultures identified by Favilli in this manner and later sent to the writer for confirmation by other methods were found to be in agreement. The second, that of Valenti, appears to be valueless. We have not been successful in our efforts to confirm his findings. As a matter of fact, we have labored for 2 years to obtain a serum possessing bactericidal properties for Brucella and our efforts have been negative.

The McAlpine and Slanetz[3] method, based on differences in the glucose metabolism of Brucella, is satisfactory for identifying *Br. abortus,* but it does not with a certainty distinguish *Br. melitensis* from *Br. suis.*

The differential method which has received the greatest amount of attention is that of agglutinin adsorption. This is uniformly valuable in distinguishing *Br. melitensis* from *Br. abortus,* but the usual technic employed does not distinguish *Br. suis* from *Br. abortus.* Henry[4] states that if R strains of *Br. suis* are used as antigen, this species may be distinguished from *Br. abortus.*

The value of any method for distinguishing closely related species of microörganisms lies in the ease and accuracy with which it can be employed and the consistency of results, even after the organism has undergone physical changes such as from an anaerobic to an aerobic state and variations in agglutinability. When more than one species occurs in infective material, such as blood or feces from cases of undulant fever, and milk or aborted fetuses from infected cattle, the differential method should tell us this fact, and further should separate one from the other. It should also find useful application in keeping laboratory cultures correctly identified.

There is another angle to the differentiation of strains of Brucella from humans alone which is assuming economic importance. This applies to those people in industries who when ill are entitled to disability compensation under the Workmen's Compensation Act if it can be

shown that the disability was acquired in the line of occupation. For example, if a worker contracts undulant fever while employed in an industry which involves the handling of animals, let us say hogs, and he seeks disability compensation, he will find that before his claim is approved, it will either be necessary for him to prove that he handled carcasses infected with Brucella, which will be impossible owing to the lapse of time between exposure and the onset of symptoms, or that the strain with which he is infected is the one common to hogs. In addition he will also have to produce proof that he has not been working with other animals or has not used raw products from cows. This is not a theoretical example. Several cases of this nature have already been brought up where it was necessary to bring forth evidence to prove that the disability, undulant fever, was contracted in line of duty.

It is firmly believed that the method[10] discussed below, which the writer has studied for the past 4 years, possesses all of the qualifications which one would desire for differentiating organisms within a genus. It is based on the growth inhibiting or bacteriostatic action of certain dyes when introduced into a medium suitable for the growth of Brucella. It has been employed in studying 656 strains of Brucella as to species. These strains were isolated from cattle, hogs, horses, goats, fowls, and humans, and came from all parts of North America, Europe and Africa.

It has been necessary to make slight changes in the technic, not because the method was faulty but to overcome possible discrepancies which seem to have had the faculty of creeping in as the study progressed. Many criticisms have been raised concerning the accuracy of the method. Some are justifiable, and it has been necessary to study and answer those. We have learned that dyes of certain manufacturers are not suitable for this technic; also, that only beef liver infusion agar of the correct formula gives accurate results. We also know that through long exposure or the incubation of plates containing the dyes in a room where there are reducing gases present, such as a 37° C. incubator, the dyes are reduced and their bacteriostatic action is impaired. The lower the concentration of a dye or the more it is reduced, the less effect will it have in inhibiting the growth of Brucella.

The differentiation method has been based on the employment of such dyes as thionin and either methyl violet or basic fuchsin. Thionin in a suitable final dilution completely inhibits the growth of *Br. abortus,* but not that of *Br. melitensis* or *Br. suis.* Methyl violet or basic fuchsin in a suitable final dilution will completely inhibit the

growth of *Br. suis* for 72 hours, but only slightly reduce the growth of *Br. melitensis* or *Br. abortus*.

In using thionin, it is important that the 1 per cent aqueous stock solution be thoroughly shaken just before it is placed in the medium, because the dye does not make a very stable solution in water. This is also true of basic fuchsin. If too long a time intervenes between shaking and placing it in the medium, the final concentration will be too low, and satisfactory inhibition of growth will not be obtained. The most satisfactory final dilution for thionin is 1–30,000, and for basic fuchsin 1–25,000.

We have also sought and found a dye possessing more uniform and constant bacteriostatic action against *Br. suis* than any of those of the triphenylmethan series. This dye is formo-rhodamine hydrochloride, commonly known as pyronin. Pyronin is soluble, and when made up in a 1 per cent aqueous stock solution, is stable. The final dilution which should obtain for completely inhibiting all strains of *Br. suis* is 1–200,000. By using different final dilutions of pyronin it is quite satisfactory for separating each of the 3 species. A final dilution of 1–100,000 permits only a slight growth of *Br. melitensis*, while all strains of *Br. abortus* give a luxuriant growth at this dilution. The stock solutions of all the dyes spoken of should be prepared by sterilizing the distilled water and dye powder separately before putting them together.

TABLE I

THE SOURCE AND CLASSIFICATION OF THE SPECIES OF BRUCELLA ACCORDING TO BEHAVIOR TOWARD DYES

Source	Number of Strains	Species		
		Br. melitensis	*Br. abortus*	*Br. suis*
Human	236	73	67	96
Bovine	263	4	249	10
Porcine	55	0	0	55
Caprine	35	35	0	0
Equine	16	0	11	5
Avian	2	0	2	0
Unknown	49	21	23	5
Total	656	133	352	171

Thus far, the dye method of distinguishing the species of Brucella has been extended to 656 strains* isolated from man and animals in

* Since the presentation of this paper a strain of *Br. suis* has been isolated from the testicle of a dog sent to this laboratory by Drs. Case and Planz of Akron, O.

continental Europe, Great Britain, Africa, Maltese Islands and North America (Table I). The strains of *Br. melitensis* from man and the goat were, for the most part, isolated in European countries. It is of interest to note that the goat has not yielded any strains of the other 2 species of Brucella. The 4 bovine strains of *Br. melitensis* were isolated from cattle in the United States and Switzerland. The 67 human strains of *Br. abortus* were from cases of undulant fever in the United States, Rhodesia and north European countries such as Denmark, Germany, and Sweden. The 11 equine strains of *Br. abortus* were, with 2 exceptions, isolated from cases of fistula of the withers in horses and mares by Dr. C. P. Fitch[5] of the University of Minnesota and Dr. Van der Hoeden[6] of Utrecht, Holland. The 2 avian strains of abortus were from fowls in naturally infected flocks in the State of Michigan. The 96 strains of *Br. suis* were from cases of undulant fever occurring in the United States. No cases of undulant fever in any European country have thus far yielded a culture of *Br. suis*. The bovine, porcine, and equine strains of *Br. suis* were isolated in the United States. It would appear that hogs are refractory to infection by *Br. abortus* since not a single strain from this source has come into the hands of the writer. The 49 unknown strains were sent to the laboratory without any history. Many of these were isolated several years ago and had passed through several laboratories before reaching ours.

DISCUSSION

If the method of identifying the species of Brucella according to their behavior toward dyes is correct, it would seem that one can no longer doubt that *Br. abortus* is one of the causes of undulant fever in America and north European countries. This method substantiates the epidemiological data collected by students of the disease here and abroad.

The comparative pathogenicity of *Br. abortus* and *Br. suis* for man has been based largely on the number of successful recoveries of each in blood cultures. It is a well known fact that numerous cultures have been taken in cases of the disease with negative results. It has also been demonstrated that *Br. suis* can be obtained in culture from the blood with a greater percentage of success than can *Br. abortus*. Therefore, it is perfectly evident that at present one cannot say that *Br. suis* is the predominating species in undulant fever in America just because a greater number of isolations have been obtained. If *Br. abortus* is as pathogenic for man as the epidemiological and laboratory findings indicate, there is still to be answered the important question which has been raised many times: Why is the number of cases of

undulant fever due to *Br. abortus* so out of proportion to the number of individuals that have been exposed to infective material?

It is a well known fact that, of the thousands who take *Br. abortus* into their bodies when they consume infective raw milk or cream, a very small proportion develop undulant fever. The failure of many to become infected may be attributed to a native immunity to Brucella. Another hypothesis that has been advanced for the low incidence of infection as compared with the numerous exposures is gradually becoming a reality. Favilli[7] first offered the suggestion that there might be a wide difference in the virulence of strains of *Br. abortus* and that possibly only a certain strain was pathogenic for man. Meyer and Eddie[8] also made the same suggestion based on their difference in pathogenicity for monkeys.

We have observed that strains of *Br. abortus* differ markedly in their ability to reduce basic fuchsin in beef liver agar. Those that have been studied appear to divide themselves into 2 groups: one, when grown on plates of the dye medium for 72 hours, produces a zone of complete decolorization, which extends outward and around the growth to a distance of 5 to 15 mm.; the other, grown for the same length of time, produces only a slight decolorization. This difference in dye reducing ability is true of newly isolated CO_2 anaerobic strains as well as older aerobic ones. It has also been observed that those strains which decolorize the dye slightly rarely, if ever, produce the abundant growth that is produced by the intensely decolorizing ones. The interesting feature about the 2 groups is that those strains which are not active reducers of basic fuchsin appear to come from humans and cattle, while those that completely reduce the dye come only from cattle. About 80 per cent of the strains from cattle that have been studied belong to the latter group.

One of the problems that has interested us in connection with the study of the behavior of Brucella toward dyes and which we have spent considerable time in trying to solve, is the nature of the bacteriostatic reaction. Our data (to be presented in detail later) show that Brucella possesses a very active reducing system which operates on the food in the medium to make it available for the metabolism of the cell. The activity of the reducing system varies with the species of Brucella. When these particular dyes are added to the medium, they combine with the food, thus preventing or retarding its reduction, and prevent or retard the natural metabolism of the cell. In order for Brucella to grow, its reducing system must affect one or more of the dyes. *Br. suis* possesses a very actively reducing system for thionin, but not for fuchsin or pyronin. *Br. abortus* is able to reduce fuchsin and pyronin,

but not thionin in certain concentrations. *Br. melitensis* can reduce each of the dyes in question. Its reducing activity, however, is lower for pyronin than is that of *Br. abortus*. If one artificially maintains the dyes in a reduced state in the medium, there will be no inhibition of the growth of any Brucella.

Besides the identification of the species of Brucella, the dye method, combined with the determination of the activity of H_2S metabolism, plays an important rôle in separating two species when accidentally mixed or when they occur together in a single blood culture from a case of undulant fever. Dr. Borts, of the Iowa State Board of Health Laboratories, has twice separated from a blood culture *Br. abortus* and *Br. suis* and once *Br. abortus* and *Br. melitensis* by use of the dye method.

They are also useful in separating Brucella from bacteria of another genus that appear like Brucella in their morphological and cultural characteristics and cross agglutinability. One particular organism which the writer has in mind, and which could easily be confused with *Br. suis,* is *Pfeifferella mallei.* The natural host of this organism is the horse. So also is *Br. suis* sometimes found in the horse. The lesions produced in the guinea pig by both organisms through subcutaneous or intraperitoneal inoculation are not entirely unlike in character. Both attack the same organs, producing large necrotic abscesses, and cause abscess formation of the joints of the extremities. *P. mallei* is agglutinated by serum prepared against *Br. suis,* and *Br. suis* is agglutinated by a *P. mallei* antiserum. The reducing activity of *P. mallei* for dyes differs from that of *Br. suis* in that it gives a luxuriant growth on a medium in the presence of either thionin, basic fuchsin or pyronin. *P. mallei* is also a very active H_2S producer. This gas is produced in large amounts over a period of 10 to 15 days' incubation as determined by lead acetate paper. *Br. suis* produces very little H_2S after the 4th day of incubation. Cultures of supposed Brucella or *P. mallei* from horses should be examined very carefully by laboratory methods in order to avoid an erroneous classification which later might be disastrous.

The dyes which inhibit the growth of Brucella are now being found suitable as therapeutic agents for the successful treatment of undulant fever. Leavell, Poston and Amoss[9] have found that the daily oral administration of small doses of the dyes brings about a complete recovery in chronic or resistant cases. Thionin is administered when *Br. abortus* is involved and methyl violet when *Br. suis* is the infecting species.

Pyronin should prove even of more value as a therapeutic agent

than the two dyes just mentioned because in low dilutions it is effective against all three species, and, if given slowly, it may be injected intravenously in a 1 per cent solution without harmful effects. Monkeys easily tolerate from 10 to 20 c.c. by the intravenous route.

SUMMARY

A total of 656 strains of Brucella from Europe and North America have been studied in their growth behavior toward aniline dyes in a suitable medium.

It has been found that these strains divide themselves into 3 groups or species according to the growth inhibiting action of thionin in a final dilution of 1–30,000, and pyronin in a final dilution of 1–200,000 in beef liver infusion agar at a pH of 6.6.

Of the total number, 133 have been classified as *Br. melitensis* (Bruce), 352 as *Br. abortus* (Bang), and 172 as *Br. suis* (Traum). *Br. melitensis* grows on both the thionin and pyronin dye medium, while *Br. abortus* grows only on the one containing pyronin and *Br. suis* only on the one containing thionin.

The strains of *Br. abortus* may be divided into 2 classes: those which completely reduce basic fuchsin (final dilution 1–25,000 in beef liver infusion agar), and those which slightly reduce this dye. It appears, on the basis of isolated cultures, that the latter class is the one that is pathogenic for man.

When more than one species of Brucella occurs in blood culture or other infective material, the dye method and H_2S metabolism determination will separate and identify each of the species.

The growth inhibiting action of these dyes, especially pyronin, offers possibilities in the therapeusis of undulant fever in man and Brucella infections in animals.

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